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Enhanced cGMP-Dependent Signaling in Astrocytes: Novel Therapeutic Target in Alzheimer's Disease

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ENHANCED cGMP-DEPENDENT SIGNALING IN ASTROCYTES: NOVEL THERAPEUTIC TARGET IN ALZHEIMER'S DISEASE

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ENHANCED cGMP-DEPENDENT SIGNALING IN ASTROCYTES: NOVEL THERAPEUTIC TARGET IN ALZHEIMER'S DISEASE

Abstract

Over five million Americans suffer from Alzheimer's Disease (AD), with an expected 34% increase in the incidence in this disease over the next decade. Unfortunately, there is no cure for AD. Recent studies have indicated that drugs which increase the levels of cyclic guanosine-3'5'-monophosphate (cGMP) may help preserve learning and memory in AD and enhance cognition in the aging brain; however, the mechanism(s) of how cGMP exerts this beneficial effect is unknown. The present findings now suggest that elevation of cGMP in astrocytes depresses inhibitory potassium currents in these cells to stimulate their protective influence on neuronal activity. Cellular currents were measured directly in rat embryonic astrocytes via the perforated-patch whole-cell patch-clamp technique, and these experiments demonstrated robust outward currents due to potassium efflux. Currents exhibited slowly-activating, non-inactivating kinetics, and were sensitive to inhibition by tetraethylammonium. Thus, these currents were carried predominately by the delayed rectifier potassium (K_{DR}) channel, which is highly expressed in astrocytes. Stimulation of cGMP signaling with sodium nitroprusside (a nitric oxide donor; 10mM; 10min) depressed

steady-state outward currents by an average of 34% (n=3). In other cells addition of 8Br-cGMP (500mM; a membrane-permeable cGMP derivative) also depressed these currents (18.4%, n=3). In contrast, neither SNP nor 8Br-cGMP altered rapidly-inactivating, A-type potassium currents significantly. Lastly, elevating cyclic adenosine-3'5'-monophosphate (cAMP) levels in astrocytes with forskolin (10mM, 10 min) also depressed outward currents (17%; n=3). Taken together, these findings suggest a novel cellular transduction mechanism that could contribute to the beneficial effect of NO/cGMP signaling on learning and memory: depression of K_{DR} currents, which depolarizes astrocytes, and thereby increases calcium influx via voltage-dependent (L-type) calcium channels. Increased astrocytic calcium levels would in turn excite these cells and enhance release of gliotransmitters to promote normal synaptic neurotransmission.

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1. INTRODUCTION

I. Pathology and Pathological Diagnosis of Alzheimer's Disease

Signs of AD include, but are not limited to, widening of sulci, shrinkage of the gyri, the thinning of the cortical ribbon, ventricular dilation, atrophy of the amygdala and hippocampus, significant loss of neurons, neutrophil threads, granulovascular degeneration and amyloid angiopathy (Yaari et al., 2007). Normally the brain of a patient with AD shows signs of atrophy in all areas except the occipital portion (Yaari et al., 2007). Neuritic plaques and neurofibrillary tangles are hallmark signs of AD, although they could be signs of other neurodegenerative disorders such as dementia (Yaari et al., 2007). Neuritic plaques are spherical in structure and consist of amyloid proteins surrounded by atrophic neuritis (Yaari et al., 2007). There are two other plaques that are seen in the brain of AD patients: "burnt out" and diffuse plaques (Yaari et al., 2007). Diffuse plaques consist of unstructured amyloid protein and "burnt out" plaques consist of dense core amyloid protein (Yaari et al., 2007). Amyloid protein consists of β-amyloid$_{1-42,}$ formulated from an abnormal processing of the amyloid precursor protein (Yaari et al., 2007). β-amyloid$_{1-42}$ is toxic to the brain and considered critical to the pathogenesis of AD due to the formation of insoluble clumps that lead to neuronal atrophy and death (Yaari et al., 2007). Neurofibrillary tangles, also seen in the brain of AD

patients, can also have devastating affects. Neurofibrillary tangles can cause interference with the cytoskeleton structure of neurons, which ultimately causes neuronal cell death (Yaari et al., 2007). Neurofibrillary and neuritic plaques are not distributed evenly across the brain, but rather attack important parts of the brain that effect specific neuronal systems (Yaari et al., 2007).

Pathological diagnosis of AD must meet certain criteria during examination of the brain post mortem (Yaari et al., 2007). These criteria include a significant number of neuritic plaques and neurofibrillary tangles (Yaari et al., 2007). Small amounts of neuritic plaques and neurofibrillary tangles can be found in clinically normal elderly persons. Therefore, a stricter guideline from the National Institute on Aging-National Institute of Neurological and Communicative Disorders (NIA-NINCDS) and Consortium to Establish a Registry for Alzheimer's Disease (CERAD) is deemed necessary for the pathological diagnosis of AD (Mirra et al., 1993). Quantitative analysis of age-adjusted densities and frequencies of plaques (given by NIA-NINCDS and CERAD criteria), presence of neurofibrillary tangles and clinical diagnoses of dementia are needed to diagnose AD pathologically (Mirra et al., 1993).

II. Clinical Diagnosis of Alzheimer's Disease

There are two commonly used criteria and guidelines put forth that help diagnose patients with AD: Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) and the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al., 1984). Clinical cognitive decline presentation tends to be a precursor or sign of AD. In early stages of AD, clinical features include, but are not limited to, short-term memory loss, preservation of remote memory, some difficulty with word finding, reduction in the ability to plan, judge, and organize and preserved social behavior (Yaari et al., 2007). In the intermediate stages of AD, clinical features include, but are not limited to, deterioration of logical reasoning, planning and organizing, decline of remote memory, loss of day to day skills, delusions, visual hallucinations, emotional distress and agitation, decrease in concern for appearance and depression (Yaari et al., 2007). Late stages of clinical AD consist of severe deterioration of all cognitive functions, inability to speak, uncontrolled urinary and bowel systems, involuntary jerking and require assistance with normal day-to-day activities (Yaari et al., 2007). Clinical diagnosis of AD is usually determined by excluding other diseases and disorders with the same clinical features such as manic-depressive disorder, Parkinson's disease, multi-infarct dementia, drug intoxication, thyroid disease, pernicious anemia, luetic brain disease, subdural hematoma, occult hydrocephalus,

Huntington's disease, Cruetzfeldt-Jakob disease and brain tumors (McKhann et al., 1984)(Yaari et al., 2007). Exclusion is accomplished by using brain imaging, objective neurological testing, neuropsychological testing, laboratory testing, clinical examination and functional assessments (Yaari et al., 2007).

NINCDS-ARDA created the criterion for clinical diagnosis of AD to address the substantial amount of cases diagnosed with AD that were found at autopsy to have other conditions not AD related (McKhann et al., 1984). It was important for the NINCDS-ARDA to create a uniform criterion for diagnosing AD in order to develop a meaningful response to treatment (McKhann et al., 1984). According to NINCDS-ARDA, there are three stages of clinical criteria of diagnosis of AD: *Probable*, *Possible*, and *Definite*. The criteria for the clinical diagnosis of *Probable* Alzheimer's disease consists of diagnosis of dementia via the Mini-Mental Test, Blessed Dementia Scale and confirmed by neuropsychological tests, signs of deterioration in two or more cognitive areas, progressive decline in memory and any other cognitive area, no disruption of consciousness, age must be between 40 and 90, and exclusion of other possible diseases or disorders that may be the cause of decline in the cognitive areas aforementioned (McKhann et al., 1984). The criteria for the clinical diagnosis of *Possible* Alzheimer's disease can be made on the basis of dementia syndrome if all other possible diseases or disorders are absent (McKhann et al., 1984). Secondly, if there is a presence of a second systemic or brain disorder not considered to be the cause of dementia then the patient can be diagnosed as *Possible* (McKhann et al., 1984). The criteria for the clinical diagnosis for

Definite Alzheimer's disease includes the clinical criteria for *Probable* Alzheimer's disease and histopathological evidence obtained post mortem.

Standard methods of examination via medical history, neurological tests, psychiatric tests, clinical examinations, neuropsychological tests and laboratory studies are needed to determine the clinical diagnosis stage of AD based on the criteria provided by the NINCDS-ARDA (McKhann et al., 1984). Medical history is taken to determine the progression of deterioration and to identify what tasks the patient is no longer able to perform adequately (McKhann et al., 1984). The medical history can also be taken from an individual that is familiar with the routines of the affected patient to document changes in various functions that the patient may not be aware of such as the inability to use money, difficulty in dressing, reading and writing and the inability to recognize previously familiar individuals (McKhann et al., 1984). Clinical examination is used to document symptoms of cognitive impairment, depression, hallucinations, dementia via mini-Mental State Examinations or Blessed Dementia Scales and impairment of sensory and motor systems (McKhann et al., 1984). This complete examination allows the physician to exclude other neurological disorders and disease. Neuropsychological testing is used to gain additional information for a complete and thorough diagnosis of dementia, and is valuable for determining the patterns and progression of impairment over a period of time after therapies have been instituted (McKhann et al., 1984). Specific laboratory tests are used to enhance diagnostic accuracy by identifying other causes of dementia (McKhann et al., 1984). There are currently no

present laboratory tests that can diagnose AD during the life of the patient (Yaari et al., 2007). It is known that AD patients tend to show increased slow-wave activity that becomes more marked with the progression of the disease. Electroencephalography is used to measure the wave activity of patients believed to have AD, and is used as an electrophysiologic method to determine if dementia is caused from depressive syndromes (McKhann et al., 1984). Computerized tomography is used to exclude diseases and disorders such as subdural hematoma, brain tumor, hydrocephalus, and dementia associated with vascular disease to further aid in the diagnosis of AD (McKhann et al., 1984). Regional cerebral blood flow (rCBF) measurements can be used to differentiate dementia from AD (McKhann et al., 1984). A decreased measurement of regional cerebral blood flow is seen in patients with Alzheimer's disease (McKhann et al., 1984). Quantitative assessment of the rate of glucose utilization, oxygen consumption, and rCBF is assessed through positron emission tomography and used to determine if the patient's levels are concurrent with encephalomalacia or AD (McKhann et al., 1984). Magnetic Resonance Imaging is also an important laboratory test due to its ability to reveal demarcations of gray and white matter which is helpful in delineating differences between AD and multi-infarct dementia (McKhann et al., 1984). Although laboratory test are not being used to diagnose AD, some markers and histopathologic hallmarks related to neuritic plaques and neurofibrillary tangles are used to improve diagnostic accuracy (Yaari et al., 2007).

III. Basic Function of Astrocytes

Astrocytes play a vital role in brain function, and are the major glial cell population in the CNS (Brahmachari et al., 2006). Radial glial cells serve as the precursor and direct descendants of astrocytes that formulate the grey matter of the brain (Bushong et al., 2004). Astroglial syncytium is a physical integrated network that connects adjacent astrocytic cells via gap junctions that allow long-range signaling (Verkhratsky et al., 2010). Astrocytes are involved in sending processes to blood vessels, in turn making up the neurovascular unit which provides metabolic support in the brain (Verkhratsky et al., 2010). Astrocytes also maintain cellular homeostasis of blood flow, extracellular fluid, ions, transmitters, and regulation of synaptic function (Brahmachari et al., 2006). Because of the multifunctional roles astrocytes play in neuronal protection, it is likely that gaining a better understanding of astrocyte function will lead to a clearer understanding of their role in Alzheimer's disease – and as a potential therapeutic target.

Astrocytes provide a metabolic connection between blood vessels and the parenchyma of the brain, thus constituting a neurovascular unit (Verkhratsky et al., 2010). There are two steps needed to maintain the metabolic support of the neurovascular unit. First, astrocytes use neuronal activity to regulate local blood flow, which is established by rapid vasodilation after a local increase in neuronal firing (Verkhratsky et al., 2010). This

increased synaptic transmission causes astroglial Ca^{2+} signaling to trigger the release of vasoactive substances (Ladecola et al., 2007). Secondly, astrocytes provide metabolic substrates during increased glutamate release, which activates Na^+-dependent glutamate transporters (Verkhratsky et al., 2010). This causes an increase in intracellular Na^+ concentration in astrocytes triggering glycolysis and lactate synthesis (Verkhratsky et al., 2010).

Extracellular ion concentration is extremely important in maintaining brain function, and astrocytes play a major role in this process. Potassium ions are one of the most important ions in considering homeostasis in the extracellular space of the brain due to the large quantities released during neuronal activity (Verkhratsky et al., 2010). Increased extracellular potassium can depolarize the neuronal membrane, which alters excitability (Verkhratsky et al., 2010). Astrocytes in the brain maintain homeostasis of extracellular potassium concentrations through spatial K^+ buffering (Kofuji et al., 2004). When extracellular potassium concentrations are at a higher level than normal, inward rectifying potassium channels provide an influx pathway for excess extracellular K^+ to reestablish normal extracellular concentrations (Verkhratsky et al., 2010). Spatial K^+ buffering is accomplished by the relocalizing of K^+ ions from regions of high K^+ concentrations to regions of low K^+ concentrations within the local astrocytic network via astroglial water transport (Simard et al., 2004).

Astrocytes are also important in neurotransmitter homeostasis in the brain (Verkhratsky et al., 2010). Glutamate is the main excitatory

neurotransmitter released into the extracellular space of the brain during synaptic transmissions (Verkhratsky et al., 2010). Removal of glutamate is important for preventing excitotoxicity and is taken up by perisynaptic astrocytes (Swanson et al., 2005). Non-toxic glutamine is formed from glutamate after it is taken up by perisynaptic astrocytes and transported back to the presynaptic terminal where it is converted back to glutamate for use in synaptic transmission (Martinez-Hernandez et al., 1977 V39).

Astroglia's role is to preserve the integrity of the host tissue following an injury. Atrophic astrocytes are called reactive astrocytes (Ridet, J. et al., 1997). Hypertrophy and proliferation of microglial cells and astrocytes are considered reactive gliosis (Ridet, J. et al., 1997). Studies have shown that reactive gliosis may prevent axonal regrowth but isolates CNS from further damage (Ridet, J. et al., 1997). However, the pathology of reactive astrocytes in neurological disorders remains far from being fully understood (Ridet, J. et al., 1997).

IV. The Role of Astrocytes in Alzheimer's Disease

Alois Alzheimer discovered an abundance of glial cells in neuritic plaques. Neuroglia plays a role in determining progression and outcome of most neurological diseases (Verkhratsky et al., 2010). As discussed earlier, neuroglia is important for homeostasis in brain function and any disruption in homeostasis can be fatal for the nervous system. Astroglia are normally affected during the early stages of neurodegenerative disease, and may lie at the core of initial disruption of neural circuitry in atrophic changes. Reactive astrogliosis has become a hallmark of plaque-infested brains in the late stages of AD (Nagele et al., 2003). Reactive astrogliosis is initiated by an increase in the number of astrocytes due to the obliteration of local neurons from neurodegenerative atrophy (Kofuji et al., 2003).

Increased astrogliosis has shown to have a correlation with increases in cognitive decline (Simpson et al., 2010). Reactive astrogliosis in AD can be initiated by β-amyloid peptide (AB) along with many other factors. Astrocyte exposure to AB causes changes in Ca^{2+} signaling which contribute to astroglial neurotoxicity (Abramov et al., 2003). Studies have shown that astrocytes within AD-like conditions express BACE-1 (beta-site APP cleaving enzyme-1), which gives the ability to produce AB (Rossner et al., 2005). Development of AB in the brain of patients with AD requires two sequential cleavages of the APP (amyloid precursor protein) done by BACE-1 (Jonsson et al., 2012).

Studies show that patients in the early stages of AD are found to have vascular impairments leading to a reduction in blood flow (Bell et al., 2009). Astrocytes play a major part in the neurovascular unit controlling the link between neuronal activity and local blood flow through several signaling cascades. In AD, the neurovascular unit is a potential target of dysfunction because of the presence of brain capillaries within AB plaques, which affects microcirculation and vascular AB clearance (Bell et al., 2009). Astrocytes control excess blood flow (hyperemia) and local cerebral circulation, which is important in functional activity of neural networks (Verkhratsky et al., 2010). Atrophic astrocytes may cause dysfunction of the neurovascular unit; however, the mechanism remains unclear in AD.

Early stages of AD in humans are characterized by a significant decrease in glucose use, thus causing a metabolic remodeling of astroglia in AD (Verkhratsky et al., 2010). Studies show that AB causes this remodeling by increasing reactive oxygen species production in astrocytes (Verkhratsky et al., 2010). However, the glucose metabolic pathways in AB cultured astrocytes are not fully understood. There are many discrepancies amongst studies that show an increase and decrease in the activity of enzymes associated with glucose metabolism, which may be due to cell-specific changes in glucose metabolism developing at different stages of AD.

Atrophic astrocytes causes reduced synaptic coverage, dysfunction of neurovascular unit, reduced functional hyperemia, reduced metabolic support, and altered extracellular homeostasis of ions and neurotransmitters which can

be the source of reduced synaptic connectivity through synaptic malfunction leading to cognitive decline in early stages of AD (Verkhratsky et al., 2010). AB plaque causing reactive astrogliosis leads to the release of inflammatory and neurotoxic factors, which impairs homeostatic function (Verkhratsky et al., 2010). Dysfunction of normal homeostatic brain function leads to neuronal death, and causes cognitive decline and atrophy of the brain in the late stages of AD.

V. Glial Fibrillary Acidic Protein Expression in Astrocytes

As mentioned above, reactive astrocytes may play a key role in Alzheimer's disease and many other neurodegenerative diseases. The secretion of neurotrophic factors in astrocytes is used for neuronal survival, but the rapid overexpression of these factors ultimately leads to neuronal death and brain injury (Brahmachari et al., 2006). Glial fibrillary acidic protein (GFAP) is one of the many factors expressed in astrocytes, and is localized to astrocytes in the brain based on immunofluorescence and histological studies (Bignami et al., 1977). GFAP is often used as a tool to monitor development and locate reactive astrocytes within the brain via staining techniques in vitro and in vivo (Morrison et al., 1985). Studies have shown that when astrocytes become reactive, there is an increased expression of GFAP (Brahmachari et al., 2006). Studies also show that Alzheimer's pathologic hallmarks, senile plaques, are linked with GFAP-positive reactive astrocytes (Brahmachari et al., 2006). The increased levels of GFAP are synonymous with the severity levels of astrogliosis (Brahmachari et al., 2006). However, there is no evidence that supports the actual mechanism that shows how increased astroglial expression of GFAP affects neurodegenerative disease (Brahmachari et al., 2006).

VI. Function of K^+ Channels in Potassium Spatial Buffering

Regulation of extracellular potassium concentration is extremely important for proper neuronal function (Bay et al., 2012). Potassium efflux occurs from axons during action potential propagation causing an increase in extracellular potassium concentration (Somjen et al., 2001). Uncontrolled increase of K^+ extracellular space can lead to the disruption of neuronal activity (Chever et al., 2010). The regulation of extracellular potassium concentration at synaptic sites is controlled by a mechanism referred to as potassium spatial buffering or potassium siphoning (Kofuji et al., 2004). The membrane potential of astrocytes at high neuronal activity is negative to the potassium Nernst potential causing a driving force to influx extracellular potassium ions (D'Ambrosio et al., 2002). Extracellular potassium ions in the brain flow through potassium inward rectifying channels (Kir), more specifically the Kir4.1 channel, resulting in potassium clearance (Bay et al., 2012). Once the uptake of potassium has occurred, the potassium is then redistributed to other astrocytes with low potassium activity via gap junction coupling or indirect coupling of astrocytes (D'Ambrosio et al., 2002). Glial cells are considered to have a high conductance of potassium ions (Higashi et al., 2001).

Kir channels consist of two membrane domains, M1 and M2, and a conserved pore segment subunit containing H5. (Abraham et al., 1999). The H5 segment of the pore and M2 subunit of the membrane are essential for

potassium diffusion (Abraham et al., 1999). Kir channels' hallmark is inward rectification in the current-voltage relationship that limits potassium efflux at depolarizing membrane potentials (Nichols et al., 1997). Kir channels are essential in potassium homeostasis that control resting membrane potential and coupling of the metabolic cellular state during membrane excitability (Jan et al., 1997). Kir channels have seven subfamilies, Kir 1.0 to Kir 7.0, with subsets within the subfamilies.

Kir4.1 is the subset of Kir 4.0 subfamily and the only Kir channel expressed in neuronal astrocytes (Higashi et al., 2001) (Hibino et al., 2004). Studies also show that Kir4.1 is essential in potassium buffering in astroglia cells (Djukic et al., 2007). Knock out of Kir 4.1 has shown to cause severe pathology, early postnatal lethality, severe depolarization of glia cells, and impairment of astrocyte potassium and glutamate uptake. Loss of function of Kir 4.1 can cause tumor progression and astrogliosis (Zurolo et al., 2012).

If extracellular concentration of potassium is not controlled, it can cause neuronal injury (Bay et al., 2012). Increased extracellular concentration of potassium has shown to effect neuronal excitability therefore impairing neuronal function (Djukic et al., 2007). Exposure to elevated potassium efflux can reduce neuronal firing due to depolarization-induced inactivation of sodium channels, which decreases transmitter release (Djukic et al., 2007). Studies have shown that impairment of potassium uptake can prevent long-term depression maintenance in the hippocampus due to increased synaptic activity during neuronal potentiation (Djukic et al., 2007). This reduction in clearance of

potassium in extracellular space causes an accumulation of potassium ions, which leads to synaptic augmentation (Djukic et al., 2007). Elevated levels of potassium in extracellular space can cause depolarization, which can lead to increased glutamate release from pre-synaptic cells and enhanced activation of NMDA receptors in post-synaptic cells causing a decrease in neuronal spontaneous activity (Djukic et al., 2007).

VII. NOS/NO

Nitric oxide (NO) is produced by nitric oxide synthase (NOS) using L-arginine as its substrate (Urzula et al., 2010). There are three isoforms of NOS; neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide (iNOS) (Urzula et al., 2010). All CNS cells have the ability to synthesize NO (Lincoln et al., 1997). NO carries no charge making it relatively unreactive, which allows it to freely move across membranes without being destroyed before reaching its target (Urzula et al., 2010). NO is a free radical with a short half-life. Although NO is relatively unreactive in its steady state, in the presence O_2^- it is readily reactive, which forms cytotoxic peroxynitrite anion ($ONOO^-$) (Urzula et al., 2010). This formation of $ONOO^-$ is extremely toxic and damaging to the cell. Astrocytes however, have the ability to make NO without detriment to the cell. Low nanomolar concentrations of NO can cause synthesis of cGMP. NO synthesis is disrupted under pathological conditions, which alters cGMP concentrations (Dawson et al., 1996). Studies suggest that NO synthesis is altered in the senescent brain (Law et al., 2002).

Scientists believe that NOS plays a role in memory improvement in older animals and is an integral part in the NO/cGMP signaling pathway. Studies show increased NOS activity in the aged dentate gyrus of the hippocampus in the senescent brain when compared to younger brains of rats (Chalimoniuk et al., 1998). Researchers argue that NO regulates hippocampal processes generated during spatial learning (Urzula et al., 2010). Evidence suggests that

β–amyloid activates NOS (Colton et al., 2002). iNOS has also been present in neurofibrillary tangles within neurons (Colton et al., 2002). NOS isoform expression is increased in Alzheimer's disease and may cause oxidative damage and may trigger the activation of intracellular signaling that perpetuates the process of neurodegeneration, ultimately causing cell death (Luth et al., 2001).

Even though NO has some neuroprotective features, it has been implicated in the pathogenesis of neurological disorders, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, ischaemia, and amyotrophic lateral sclerosis (Heales et al., 1999).

VIII. cGMP/ PKG (cGMP-dependent protein Kinase)

Memory is a phenomenon that is made up of acquisition, consolidation and retrieval processes (Bollen et al., 2014). Consolidation is the process of stabilizing and storing newly acquired information (Kandel et al., 2001). Molecular transformations at participating synapses are responsible for consolidation. cGMP is known to play a role in early memory consolidation and in specific neuroplasticity of long-term potentiation (LTP) (Bach et al., 1999). Studies suggest that improved memory formation is due to the enhanced cGMP cascades via de novo protein synthesis (Lu et al., 2002). This action indicates that cGMP and PKG are in concert, which may facilitate memory formation.

In the 1970's, cGMP and PKG were the topic of research in a small number of laboratories. Much research was done to determine how this signaling system was activated, what biological processes it regulated, and what role substrates of PKG played in the cellular system. In recent years, PKG has played a major role in two pathways that contribute to smooth muscle relaxation. Atrial natriuretic peptide (ANP), nitric oxide (NO) and carbon monoxide are signaling agents that lead to increased concentrations of cGMP through the activation of guanylate cyclase (Wang et al., 1997). Recent studies have shown that these signaling pathways may play a role in neuronal function and more importantly a possible therapeutic target for Alzheimer's disease. Researchers have found that the signaling pathway for cGMP may control

mediation or modulation of neurotransmitter release and uptake, neuronal differentiation and gene expression, learning and memory, brain seizure activity, and neurotoxicity (Garthwaite et al., 1995). cGMP regulates cGMP-gated ion channels, PKG, and some subtypes of phosphodiesterases (Hoffman et al., 1992). PKG phosphorylates its substrates when activated by cGMP mediated signal transduction (Matsumoto et al., 2006). However, there is little known regarding the downstream signaling events after increased intracellular concentrations of cGMP in the nervous system. In this study we aim to provide evidence that will clarify the downstream signaling events during elevated cGMP that may lead to a better understanding of this cascade in the nervous system, more specifically in astrocytes.

Recent studies have shown that PKG does play a role in certain neuronal events resulting from elevated cGMP levels. Studies have showed that PKG plays a direct role in the increasing of neuronal Ca^{+} currents, neurotransmitter release, the activation of neuronal K^{+} channels, and the presynaptic target for the retrograde messenger in LTP; leading scientists to believe that PKG plays an essential role in regulating cGMP signaling in the nervous system (Wang et al., 1997).

There are two types of PKG, type I (PKG-I) and type II (PKG-II) (Hoffman et al., 1992). PKG-I has two variants Iα and IIβ. PKG-I has been prevalent in the plasma membrane, Golgi structures, endoplasmic reticulum, and nucleus in various cells (Pryzwansky et al., 1995). PKG-I is also expressed in the cerebrum and cerebellum (Schlichter et al., 1982). However, cerebellar expression of PKG-

I is 20-40 times higher that that found in the cerebrum leading some scientists to question the specificity of PKG localization outside the cerebellum (Schlichter et al., 1982). It has been determined that non-cerebellar PKG is derived from tissue vascularization (Schlichter et al., 1982). Initial studies showed that PKG-II mRNA was detected in the cerebrum at higher levels than in the cerebellum (Uhler et al., 1993). PKG-II is mostly found in the thalamus, cortex, septum, amygdala, and olfactory bulb (El-Husseini et al., 1995). Very little levels were seen in the hippocampus and striatum (Wang et al., 1997). However, increased levels of PKG-I were found in the hippocampus and striatum as compared with PKG-II, suggesting that PKG-I may be the specific variant in the downstream signaling cascade of cGMP during improved memory and learning (Wang et al., 1997).

Although PKG is closely related to the cAMP-dependent protein kinase (PKA), the substrate specificity of these two kinases remains misunderstood. Studies show that there are not many PKG substrates and the only one found to be relatively specific for PKG is G substrate (Walaas et al., 1983). Studies have also shown that the function of PKG substrates can be grouped into the following six categories relating to signal transduction: 1) cyclic nucleotide action, 2) protein phosphatase regulation, 3) cytoskeletal dynamics, 4) intracellular calcium homeostasis, 5) ion channel regulation, and 6) GTP-binding proteins (Wang et al., 1997).

cGMP and PKG signaling can functionally regulate ion channels, specifically potassium, chloride, and sodium channels due to their influence in

regulating neuronal function (Wang et al., 1997). cGMP activates PKG, which causes Ca^{+2} activated K^{+} channels to open causing efflux of K^{+} ions resulting in hyperpolarization and inhibiting voltage-gated calcium channels (Wang et al., 1997). This mechanism is seen in the vasculature and also in rat pituitary tumor cells.

The downstream signaling mechanisms of how cGMP and PKG modulate ion channel function in astrocytes is poorly understood, therefore additional studies are needed to clarify this mechanism. NO-stimulated cGMP accumulation can enhance PKG activity in neurons and nerve terminals making it an extremely important piece to the puzzle. It is important to map out a working mechanism that fully explains the NO/cGMP-PKG signaling pathway in the CNS as a possible novel therapeutic target for memory and cognition disorders. Future studies will be needed to gather a better understanding of PKG substrates and their functionality in this pathway and its contribution to neurotransmission mediated by cGMP.

IX. NO/cGMP/PKG Pathway

Smooth muscle relaxation, platelet aggregation and disaggregation, neurotransmission, long term potentiation, and depression are all examples of signal transduction by way of the NO/cGMP pathway (Warner et al., 1994). NO was previously understood as a pollutant only due to its physical properties however, through further understanding NO can also be seen as a signaling agent (Denniger et al., 1999). NO is an excellent ligand for heme, which allows it to bind to sGC at low concentrations and effective for signal transduction of several pathways in the cardiovascular and nervous system (Denniger et al., 1999).

Nitric Oxide (NO) is a stimulator of soluble guanylate cyclase (sGC), the only proven receptor for NO. (Denniger et al., 1999). sGC catalyzes the conversion of guanosine 5' triphosphate (GTP) to cyclic guanosine 3',5'-monophosphate (cGMP) (Denniger et al., 1999). sGC is a heterodimeric protein where NO binds to the sGC heme, which activates the enzyme, thus increasing cGMP levels (Denniger et al., 1999). This rise in cGMP allows sGC to send a NO signal to the downstream elements of the signaling cascade-cGMP-dependent protein kinase (PKG), cGMP-gated cation channels and cGMP-regulated phosphodiesterase (PDE). PKG phosphorylates target proteins in response to increased cGMP production (Lohmann et al., 1997). PDE's hydrolyze cGMP to GMP (Denniger et al., 1999). cGMP-gated cation channels open in response to

increased concentrations of cGMP (Denniger et al., 1999). This signaling pathway is intimately involved in the regulation of vascular tone and platelet function. (Denniger et al., 1999). This pathway is also involved most notably in the neurotransmission, long-term potentiation (LTP), and depression within the nervous system. Recently cGMP and PDE inhibitors have been the topic of fervor as of late because of its positive effects on improving memory and learning.

Higher concentrations of cGMP are found in the brain more than any other peripheral organ (Urzula et al., 2010). Aging of the brain causes decreases in cGMP concentrations (Denniger et al., 1999). Activation of sGC and degradation by PDE's are the regulators of the concentration of cGMP (Denniger et al., 1999). Observed decreases in cGMP concentration in the aged brain has been directly linked to lower synaptic plasticity and cognitive performance (Denniger et al., 1999). PDE inhibitors have also shown to improve learning and memory in older rats.

Understanding whether sGC's expression or activity is altered by the aging process is extremely important in understanding NO/cGMP pathway's involvement in age-related brain disorders. We understand that NO/cGMP signaling pathway is deeply involved in regulating brain function. sGC level may be a major influence to age-related brain disorders. Researchers determined that cGMP levels were significantly higher in the presence of PDE inhibitors in the aged-brain, leading scientist to believe sGC is active and not the cause of cognitive decline in the senescent brain (Denniger et al., 1999). On the other

hand inhibition of sGC blocked memory formation. Studies have shown that decline in cGMP in the aged-brain may be a causative factor in cognitive decline in the senescent brain (Puzzo et al., 2006). Increases in PDE expression and activity have shown to decrease cGMP concentration due to hydrolysis of cGMP to GMP in the aged-brain (Denniger et al., 1999). Studies suggest that administration of PDE inhibitors improves object memory and increases NOS activity in the hippocampus of aged rat brains (Denniger et al., 1999). Studies have also shown that when animals induced with PDE inhibitors PKG had no effect on NOS activity, which suggests that PKG is independent of NOS activity (Denniger et al., 1999). These data suggests that PDE inhibitors could improve cognitive and memory loss performance via NO/cGMP signaling pathway in the senescent brain.

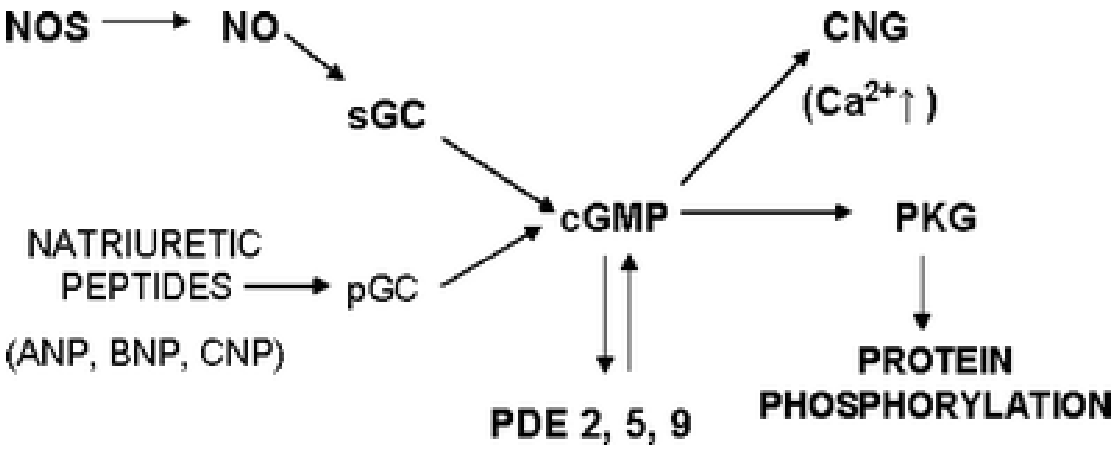

Figure 2-1

(Urszula et al., 2009)

X. Role of the NO/cGMP pathway in LTP

Studies have shown that LTP involves the activation of postsynaptic glutamanergic NMDA receptors, which is followed by an increase in calcium flow in neurons (Puzzo et al., 2006). Understanding the importance of the role of the NO/cGMP pathway in LTP in neurons can help us create a better foundation of the role this signaling pathway plays in astrocytes and its possible role in improving cognitive function.

Studies show that NOS inhibitors block LTP, which suggests that NO/cGMP pathway is extremely important in the LTP process (O'Dell et al., 1991). Large amounts of glutamate are released during LTP at the presynaptic terminals suggesting that the induction of LTP involves the production and release of NO from the postsynaptic dendrites (O'Dell et al., 1991). So given that the signaling agent NO generates cGMP, cGMP is posed to produce potentiation. Studies show that exogenous cGMP can bypass the postsynaptic events of the induction of LTP and be injected into the presynaptic neuron to produce LTP (Haley et al., 1992).

Behavioral studies have demonstrated that NO is likely to be involved in some types of learning and memory. Blocking NO via NOS inhibitor impaired spatial learning, olfactory memory, short-term memory, and long-term memory (Boheme et al.,1993). Scientist believe that NO's effect on memory is mediated through the NO/cGMP pathway (Puzzo et al., 2006).

The molecular events that encompass the NO/cGMP signaling pathway in the aging brain are of great interest to researchers as a possible therapeutic target in neurological disorders in which cognitive function declines. The ability of PDE inhibitors to restore cGMP levels, which induces calcium influx through cGMP-gated ion channels to improve cognitive performance, is a step in the right direction (Denniger et al., 1999). The molecular mechanism for why cGMP metabolism via PDE inhibitors restores cognitive function and improving memory has yet to be determined.

XI. NO/cGMP Signaling Pathway in Alzheimer's Disease

Studies have shown that Aβ peptide inhibits activation of the NO/cGMP/cAMP pathway during hippocampal synaptic plasticity (Denniger et al., 1999). Studies have also showed that NO/cGMP pathway plays a protective role in apoptosis induced by Aβ (Puzzo et al., 2006). On the other hand excessive production of NO causes neuropathology (Denniger et al., 1999). Finding the right balance in NOS production and availability without causing neurotoxicity can limit neuropathology and increase neuroprotection. This suggests that improving NO/cGMP signaling could also prove to be a novel therapeutic target for Alzheimer's disease.

Wirtz-Brugger revealed that Aβ induced apoptosis can be suppressed by NO donors, cGMP analogs, and PDE inhibitors (Wirtz-Brugger et al., 1993). Aβ also reduced production of eNOS has also been found to be a risk factor for Alzheimer's disease (McCarty et el., 1998). Induction of Aβ causes decreased production of NO because of the lack of eNOS phosphorylation (Paris et al., 1999). Aβ induced vasoconstriction can be blocked by PDE inhibition, which in turns increases cGMP levels preventing Aβ microglial inflammation and exerts a neuroprotective action via amyloid precursor protein (APP) (Paris et al., 2000, Mattson et al., 1999).

NO has also been linked with neurotoxicity in Alzheimer's disease. Studies have shown that Aβ increases the production of NO (Tran et al., 2001).

iNOS inhibitors were used as a treatment to improve spatial memory in Aβ infused rats (Haas et al., 2002). Studies showed that iNOS was increased when Aβ is increased in glial cells (Haas et al., 2002). In the microglia Aβ activates the synthesis of NO, causing neurotoxicity (Xie et al., 2002). This neurotoxicity is blocked when inhibitors of NO synthesis and catalysts of peroxynitrite decomposition are introduced to Aβ induced activated microglia in Alzheimer's disease (Xie et l., 2002).

Aβ decreases sGC expression in brain astroglial cells reducing cGMP production in patients with Alzheimer's disease (Baltrons et al., 2002). Stimulating sGC increases cGMP levels and can reestablish normal synaptic plasticity following its block by Aβ (Puzzo et al., 2006). This was confirmed by experiments in which 8-Br-cGMP, a membrane-permeable cGMP analogue, reversed the inhibition of LTP caused by Aβ (Puzzo et al., 2006). Increases in cGMP were found to reduce Aβ-induced cell death (Walsh et al., 2000). Findings suggest that KT5823, a PKG inhibitor, blocked the beneficial effect of cGMP analog 8-Br-cGMP (Puzzo et al., 2006). This lead researchers to believe that Aβ–induced LTP impairment is likely mediated through the activation of PKG (Puzzo et al., 2006). Cyclic AMP response protein (CREB) phosphorylation, a transcriptional factor, is a consequence of PKG activation that is involved in memory and learning (Bourtchuladze et al., 1994). Aβ blocks the increase of CREB phosphorylation during synaptic plasticity (Tong et al., 2001). Increasing cGMP to normal levels causes CREB phosphorylation to be reestablished. Inhibition of any of the components of NO/cGMP/PKG pathway suppresses

CREB phosphorylation (Puzzo et al., 2006). CREB phosphorylation and LTP is specifically mediated through PKG because treatment with an inhibitor of PKA did not overturn the positive effects of cGMP analogues (Puzzo et al., 2006).

Acetycholinesterase inhibitors, a common therapy for AD, have limited efficacy in improving learning and memory. Researchers are trying to find a way to inhibit the formation of neurofibrillary tangles, decrease Aβ load in the brain by use of agents that inhibit Aβ oligomerization, inhibit inflammation and oxidative damage, or by use of immunizations that remove Aβ from the brain all together. However there has been no way to implement these strategies in normal physiological conditions effectively and safely. However, drugs focusing on the NO cascade can possibly be an effective way to counteract the progression of Alzheimer's disease by acting at a level downstream of Aβ production. Results have shown that drugs that up-regulate NO can be used as a prophylactic measure to not only offset cognitive impairment and the inhibitory effects of Aβ, but also delay the natural progression of the disease. Gaining a fundamental understanding how molecular components of Alzheimer's disease affects NO/cGMP/PKG signaling cascade will be instrumental in improving upon current research.

XII. Phosphodiesterase (PDE)/ PDE Inhibitors)

Decline in cognitive function has been one of the many problems that arise as people age. Cognitive decline is also seen as a symptom in many neuropathological conditions such as Alzheimer's disease. A considerable amount of research has been done to combat cognitive decline however there is still a great need for drugs that counteract this deterioration. There has been an increase in interest in drugs that inhibit phosphodiesterase enzyme (PDE). Studies show that inhibiting PDE improves cognitive performance in animals and in man.

PDE's are enzymes that play a role in the NO/cGMP/PKG signaling pathway by hydrolyzing cGMP or cAMP into their respective inactive form throughout the body. There are 11 gene-related families of isozymes that encompass the PDE family, PDE 1- PDE 11 (Heckman et al., 2015). They are distinguished based on their affinity for cAMP and cGMP (Heckman et al., 2015). There is a strong interest in PDE 2 due to its high expression in the limbic system and the adrenal cortex (Xu et al., 2015). These specific areas are associated with cognitive function and regulation (Xu. et al., 2015). There has also been interest in PDE 3, PDE 5, PDE 6 and PDE 9 due to the high affinity for cGMP (Heckman et al., 2015).

PDE inhibitors (PDE-Is) have been of interest because of its positive effects on cognitive function when used as a therapeutic application. Impaired memory function is linked to several neurological brain disorders. PDE-Is are being used to target cognitive domains in the brain (Bollen et al., 2014). PDEs are found to be in abundance in the brain particularly in the thalamus, striatum, and prefrontal cortex (Bollen et al., 2014). Some argue that PDE-Is induced cognition enhancement is the result of increased blood flow due to PDE-Is being a vasodilator (Bollen et al., 2014). Studies suggest that PDE-Is, such as vardenafil, sildenafil, and rolipram, did enhance memory performance but did not increase blood flow in the hippocampal areas disputing earlier notions about vasodilation (Bollen et al., 2014). PDE-Is ability for memory improvement is dependent on PKG (Matsumoto et al., 2006). cGMP-PKG signaling causes Ca^{2+} influx, because of the lowered stimulation threshold for Ca^{2+}, therefore enhancing postsynaptic signaling (Bollen et al., 2014). This mechanism is thought to be the underlying effector of long-term plasticity in LTP and memory formation.

PDE-Is therapeutic use has important implications in furthering the potential treatment plan in a wide array of disorders involving cognitive decline. Although PDE-Is have pro-cognitive effects their therapeutic use is hindered due to their adverse side effects. Studies do show that lower dose treatment of cGMP specific PDE-Is and dual substrate PDE-Is prove to be a more viable option over singular PDE inhibition treatment (Bollen et al., 2014).

Further studies are needed to determine if this treatment plan will be effective clinically.

XIII. Potassium Channel Expression in Embryonic Rat Neural Progenitor Cells

Rat neural progenitor cells differentiate into neurons, astrocytes, and oligendrocytes. Two types of voltage-activated K^+ currents, non-inactivating delayed rectifier and fast inactivating A-type, have been recorded in neural progenitor cells (Liebau et al., 2006). It is known that voltage gated K^+ channels currents are most notably seen in the development of the nervous system (Smith et al., 2008). Some tetrodotoxin sensitive Na^+ currents were seen in low density, but were insufficient to generate action potentials (Sah et al., 1997). Major current expressions seen during electrophysiological tests in progenitor cells are mostly potassium channels (Smith et al., 2008). cDNA microanalysis of embryonic rat neural progenitor cells revealed that three Na^+ and thirteen K^+ genes were found (Cai et al., 2003). These genes encoded a delayed rectifier, inward rectifying, and Ca^+ sensitive K^+ channels (Smith et al., 2008).

Whole-cell recordings of embryonic neural progenitor cells revealed robust outward currents comprised predominately of delayed rectifier and an A-type K^+ channels (Smith et al., 2008). No inward currents were detected during whole cell recordings of rat embryonic neural progenitor cells (Smith et al., 2008). Due to the minimal detection of Na^+ currents one can assume that embryonic neural progenitor cells are incapable of generating action potentials (Smith et al., 2008). Although little electrophysiology has been done on embryonic astrocytes, studies done on embryonic neural progenitor cells

provide a good source of background information that proved beneficial in our research.

XIV. Function of Voltage-Gated Ca^{2+} Channels in Astrocytes

Studies have shown that Ca^{2+} in astrocytes can modulate neuronal activity (Hayden et al., 2001). Synaptic activity causes neurotransmitter receptor activation, which in turns increases intracellular Ca^{2+} in astrocytes causing excitability (Poter et al., 1996). Extracellular accumulation of K^{+} depolarizes astrocytes to induce Ca^{2+} influx via voltage-gated Ca^{2+} channels (Duffy et al., 1996). Astrocytes modulate synaptic transmission due to glutamate release via Ca^{2+} and SNARE protein-dependent exocytosis (Araque et al., 2000). Mechanisms of how Ca^{2+}-dependent release of glutamate from astrocytes remain unclear.

Voltage-gated Ca^{2+} channels are classified into two properties, low-(T-type) and high (L-,N-,P-, Q-, and R- type) voltage-gated Ca^{2+} channels (Tsien et al., 1988). High voltage-gated Ca^{2+} channels consist of different α_1 subunits, which form the ion conducting pore, selectivity filter, voltage sensors, and interaction sites for Ca^{2+} blockers and activators (Hoffman et al., 1999). Other auxiliary subunits that formulate the voltage-gated channel Ca^{2+} modulate properties such as inactivation and channel targeting to the membrane (Gerster et al., 1999). Voltage-gate Ca^{2+} channels also play a role in intracellular protein phosphorylation and gene expression (Bading et al., 1993).

Astrocytes have many of the same signaling mechanistic pathways found in neurons. Presence of voltage-gated ionic currents, neurotransmitter-gated

ionic currents and receptor-activated intracellular second messenger systems functions in neurons by means of extracellular chemical signals (Duffy et al., 1994). Depolarization of acutely isolated astrocytes increases the intracellular concentration of Ca^{2+}, which indicates that Ca^{2+} influx may activate several protein kinases (Quandt et al., 1986). Ca^{2+} influx causes astroglial depolarization and plays a role in the common pathway in neuron-glial signaling during pathology (Duffy et al., 1994). Functional significance of voltage-gated Ca^{2+} channels was confirmed through electrophysiological, pharmacological, and physiological tests (Latour et al., 2003). Ca^{2+} voltage-gated possess many ways of regulating cellular functions and homeostasis (Latour et al., 2003).

2. Hypothesis and Specific Aims

The hypothesis of the study is: Increasing cGMP depresses delayed rectifier potassium (K_{DR}) currents in astrocytes via cGMP-dependent phosphorylation, which in turn improves normal synaptic neurotransmission.

Specific Aims

1. Measure membrane currents in astrocytes directly with the perforated-patch, patch-clamp technique.
2. Identify the predominant K^+ channels carrying outward current in astrocytes.
3. Measure the effect of NO & cGMP on K^+ currents in astrocytes.
4. Determine the primary molecular target (ion channel) of cGMP in these cells.
5. Delineate cGMP signal transduction in astrocytes.

3. Methods and Materials

I. Cell Culture

Astrocytes were purchased from Neuromics. Astrocytes were received in a vial along with hippocampal tissue. The vial was placed in a sterile hood for 15 min to allow all tissue and cells to settle to the bottom. Papain (digesting solution) and Neurobasal media were placed in the incubator (5% CO_2, 37°C, humidified) for 30 min. One ml of medium was removed from the vial. Using a 1 ml pipettor and a sterile tip the tissue and remaining media was triturated in the vial until all tissue was dispersed. All material from the vial was removed and dispensed into the tube containing papain. The tube was then placed into the incubator and gently shaken every 5 min for 30 min. The solution was triturated again (10-15 times) then 1 ml of media was added to dilute the digesting enzyme solution. The solution was triturated again (10-15 times). The tube was then centrifuged at 1100 rpm for 1 min. The supernatant was removed and the Neurobasal media (6 ml) was added to disperse the pellet. The solution was triturated (10-15 times) and then the remaining 6 ml of Neurobasal was added. The solution was triturated once more (10-15 times). The cells were separated into 3 25cm^2 poly-D-lysine coated flasks. Media (Neurobasal) was changed every 2-3 days (5 ml per flask). Used poly-D-lysine coated flasks were coated using a 100 µg/ml poly-D-lysine and 1X PBS coating solution over night. Once the cells reached the proper confluence the media was removed and the

cells were washed with 5 ml of 1X PBS. The PBS was removed and 0.5-1.0 ml of 0.25% Trysin-EDTA (1X) was added to each 25cm^2 flask. The flask was placed in the incubator for 5 min. The bottom of the flask was gently tapped to remove the cells. Once all the cells detached from the flask 5 ml of Neurobasal was added to the flask. The solution was triturated to mix the cells and neutralize the trypsin. 5 ml of the cell solution was aliquot to 1-2 new 25cm^2 flasks (depending on the cell confluence before trypsinizing) and ~1ml of cell solution was used for patch-clamp experimentation.

II. Perforated Patch-Clamp

Astrocyte cells were placed on the stage of an inverted microscope and perfused at room temperature with artificial cerebrospinal fluid (aCSF) with the following composition: NaCl 120mM; KCl 15mM; $CaCl_2$ 2; $MgCl_2$ 1mM; D-glucose 10mM; HEPES 10mM at pH 7.4. Patch pipettes are made using capillaries of Corning Glass 7052 and the tip of the patch pipettes are filled with the following solution in mM: 85 KCH_3SO_3, 30 KCl, 5 $MgCl_2$, $CaCl_2$ mM and 10 Hepes. The rest of the patch pipette is back-filled with 200mg/ml amphotericin B. Amphotericin B produces microperforations in the cell membrane isolated within the patch pipette. These micropores provide electrical continuity between the pipette and the cell, thus allowing voltage-clamping and recording of cellular currents. An advantage of these micropores is that they are permeable to only monovalent ions. Therefore, the cell is allowed to control and maintain its own physiological calcium concentration as normal. In addition, important cellular signaling molecules (e.g., cGMP, PKG) do not diffuse out of the cell during an experiment – which occurs during standard whole-cell recording configurations. In summary, perforated-patch recordings provide accurate current measurement with only minimal current decay or loss of soluble cytoplasmic components due to cellular dialysis. Furthermore, endogenous calcium buffering is not inactivated by dialyzing cells with calcium chelators, as are required during traditional whole-cell recordings. Thus, the perforated-patch technique provides a more

accurate means of measuring whole-cell currents from a "metabolically-intact" cell.

An Axopatch 200B amplifier was used to control voltage-clamp and voltage-pulse generation and pCLAMP 9.2 software was used to acquire (Clampex) and analyze (Clampfit) the data. After establishing a low-resistance (<20MΩ) pathway for measuring cellular currents, as determined by amplifier compensation circuitry, cells were voltage-clamped at a holding potential of -90 or -50mV, and membrane currents were recorded via Clampex 9.2 data acquisition software. Drugs were added to the cell bath in various combinations to effectively test for project objectives: tetraethylammonium (TEA), K_{DR} blocker; sodium nitroprusside, NO donor; 8Br-cGMP, membrane-permeable cGMP derivative; KT5823, PKG inhibitor; forskolin, cAMP agonist. K^+ currents were measured through perforated, cell-attached patches in standard aCSF solution.

Whole-cell currents were measured in response to a 500 msec depolarizing test potential (-80 to +60mV) from a holding potential of either -90 or -50mV. Complete current (I) – voltage (V) relationships were constructed by measuring the peak steady-state outward current at each test potential, and these peak currents were then plotted as a function of membrane voltage. Summary I-V relationships were normalized as current density (current amplitude / cell capacitance) in order to account for possible variations in current amplitude due to cell size.

III. Nitric Oxide Production Can Be Stimulated In Astrocytes

The following steps were followed for detection of nitric oxide production in astrocytes. Astrocytes were split into 11 poly-D-lysine coated wells of a 24-well plate. Incubated with 1 ml Neurobasal medium for 4 days before experimentation. Neurobasal medium was removed and 1 ml of 2μM DAF-FM 4-amino-5-methylamino-2',7'-difluorofluorescein, Diaminofluorescien-FM (DAF-FM; fluorophore detecting NO production; diluted in KREBS solution) was added to 10 wells. Only KREBS solution was added to the 11^{th} well for control. Astrocytes were incubated for 45 min in the DAF-FM solution. After 45 min the DAF-FM solution was removed and 10μM sodium nitroprusside was added to 6 of the 10 wells that were incubated with DAF-FM for 10 min. Fluorescence was detected using the EVOS microscope GFP filter.

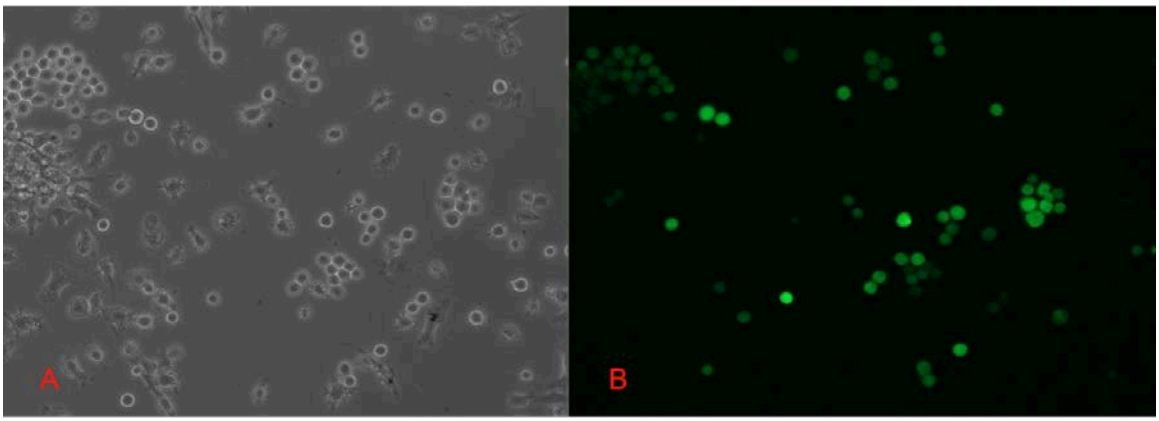

Figure 4-1

Figure 4-1 A. Imaging of cells demonstrated typical astrocytic phenotype under phase lighting. **B.** Same field of astrocytes loaded with DAF-FM and treated with 10μM sodium nitroprusside (10 min). Green fluorescence indicates production of nitric oxide in response to drug treatment.

Statistics

All data were expressed as the mean ± S.E. Statistical significance between two groups was evaluated by Student's *t* test for paired data. Comparison between multiple groups was made by the One-Way Analysis of Variance test. A probability of less than 0.05 was considered to indicate a significant difference.

4. Results

I. K_{DR} Currents Dominate Membrane Electrical Activity In Astrocytes

There are 38 different voltage-gated K^+ channel genes within the Kv family. Eleven K^+ channels are encoded as delayed rectifiers and 6 are encoded for A-type channels (Smith et al., 2008). Thus, our first task was to employ electrophysiological methods to directly identify the specific type(s) of K^+ channels, which are expressed and functional in embryonic astrocytes. Whole-cell recordings measured prominent outward currents (Figure 5-1). In contrast, no significant inward currents were detected. From a holding potential of -90mV, currents often exhibited a somewhat biphasic kinetic profile – rapid activation, followed by a slower, more sustained steady-state component. Subsequent analysis revealed this composite outward consisted of two prominent outward currents: fast-activating/inactivating current, and a more slowly activating, non-inactivating current. These currents were consistent with astrocytic expression of the fast A-type potassium current, and the slower, but sustained delayed rectifier (K_{DR}) potassium current.

Because A-type currents inactivate fairly rapidly at depolarized potentials, we were able to differentiate these 2 types of K currents by varying the holding potential. Although A-type currents are fully activated by depolarization from a holding potential of -90mV, voltage-clamping astrocytes at a depolarized holding potential of -50mV largely inactivates A-currents. Thus

by digitally subtracting the currents measured from V_{hold} = -50mV (predominantly K_{DR} currents) from the composite currents measured from V_{hold} = -90mV, we were able to isolate the underlying A-type currents (as illustrated in Figure 5-1) which contribute minimally to steady-state outward currents.

In addition to this biophysical analysis, we were able to differentiate these distinct currents by pharmacological means. Tetraethylammonium (TEA; 3mM) is generally considered a non-selective K channel inhibitor, but K_{DR} channels are sensitive to low millimolar concentration of TEA. In contrast, A-type currents are largely insensitive to TEA. We found that TEA attenuated steady-state outward currents elicited from a V_{hold} of -50mV by >50% at the peak of the I-V relation (+120 mV), and by an average of 37.0 $\pm$ 0.5% at less depolarized voltages (+100 mV; n=2). In contrast, TEA had little effect on A-type currents (as revealed by the above subtraction protocol; Figure 5-2). Taken together, these biophysical and pharmacological analyses indicate that both A-type and K_{DR} potassium channels carry outward currents in astrocytes. Further, these findings indicate that we are able to study K_{DR} currents essentially in isolation simply by employing a V_{hold} of -50mV (where A-type currents are largely inactivated).

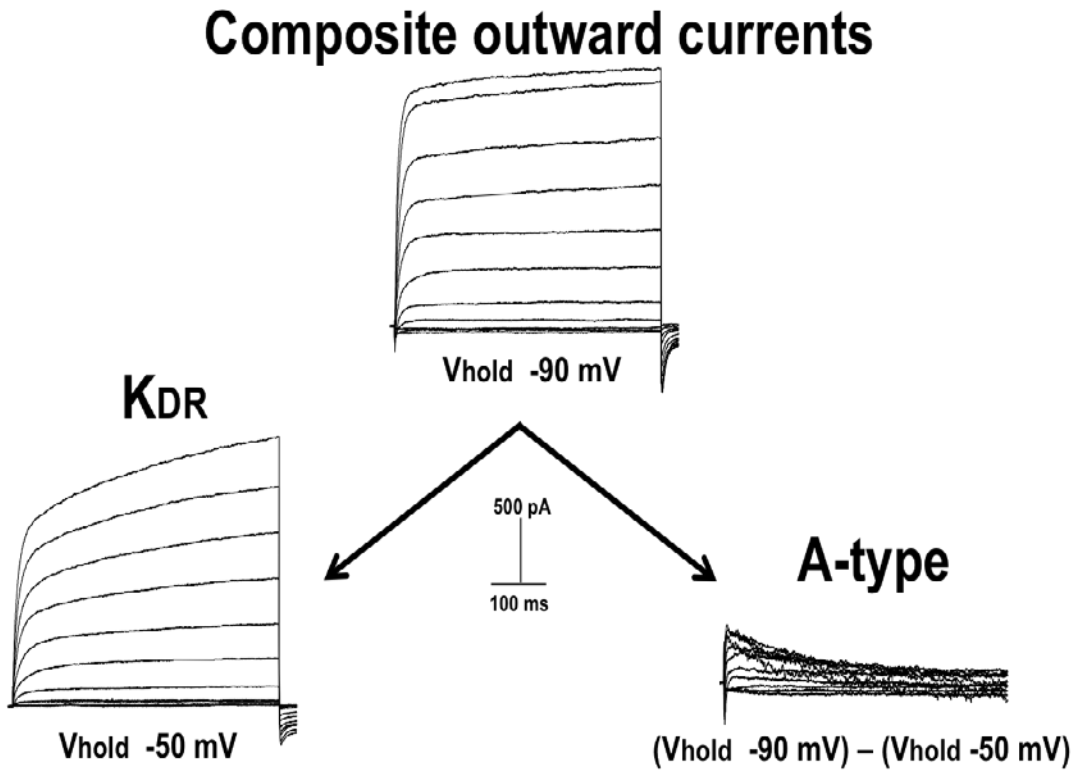

Figure 5-1

Figure 5-1: Biophysical identification of K_{DR} and A-type potassium currents in astrocytes. *Top panel:* Family of composite currents (-80 to +120mV test potentials) elicited from a holding potential of -90mV. Each test potential was a 500 msec depolarizing voltage step. *Left panel:* Family of currents elicited from a holding potential of -50mV. These currents were carried predominantly via K_{DR} channels because setting the holding potential to depolarized voltages inactivates A-type currents. *Right panel:* Family of calculated test potentials generated by digitally subtracting K_{DR} currents from composite currents to reveal underlying A-type currents.

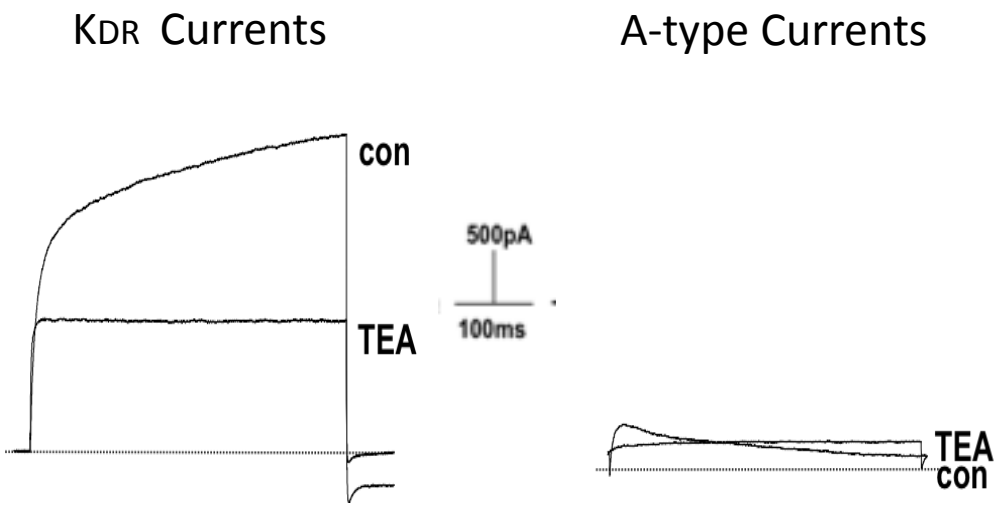

Figure 5-2

Figure 5-2: Pharmacological characterization of potassium currents in astrocytes. *Left panel:* Outward currents measured in astrocytes in response to a +120mV test pulse from a V_{hold} of -50mV. Currents were recorded before (con) and after a 15-min exposure to 3 mM tetraethylammonium (TEA). *Right panel:* Calculated A-type outward currents from astrocytes in response to a +120mV test pulse. Currents were recorded before (con) and after a 15-min exposure to 3 mM tetraethylammonium (TEA).

II. Nitric Oxide Decreases K_{DR} Currents In Astrocytes

There is increasing evidence that aging may alter how the brain responds to NO, although the effects of NO on brain function remain controversial. Moreover, little is known regarding how NO affects the ionic mechanisms governing astrocyte excitability. To investigate this mechanism, we treated astrocytes with sodium nitroprusside (SNP), which releases NO when metabolized by cellular enzymes. As illustrated in Figure 5-3, treating astrocytes with SNP reduced outward currents, carried mainly via K_{DR} channels (V_{hold} = -50mV), at all positive test voltages. On average, a 15-min exposure to 10 μM SNP reduced the density of K_{DR} steady-state currents by 33 $\pm$ 6% (+80mV test potential; V_{hold} = -50mV; n=3 cells). A summary normalized current – voltage relationship for several astrocytes, before and after SNP, is illustrated in Figure 5-3.

To our knowledge, these findings provided the first direct evidence that NO could depress K_{DR} currents in astrocytes; however, signaling events downstream from NO generation remained unknown. Because a primary target of NO action is stimulation of soluble guanylyl cyclase to catalyze the generation of cGMP from GTP, our next series of experiments tested the effects of cGMP on K_{DR} currents in astrocytes. Unfortunately, cGMP is essentially membrane impermeable. To overcome this experimental limitation we employed a more

permeable derivative, 8Br-cGMP, which diffuses across the plasma membrane of astrocytes and stimulates cGMP signaling within these cells.

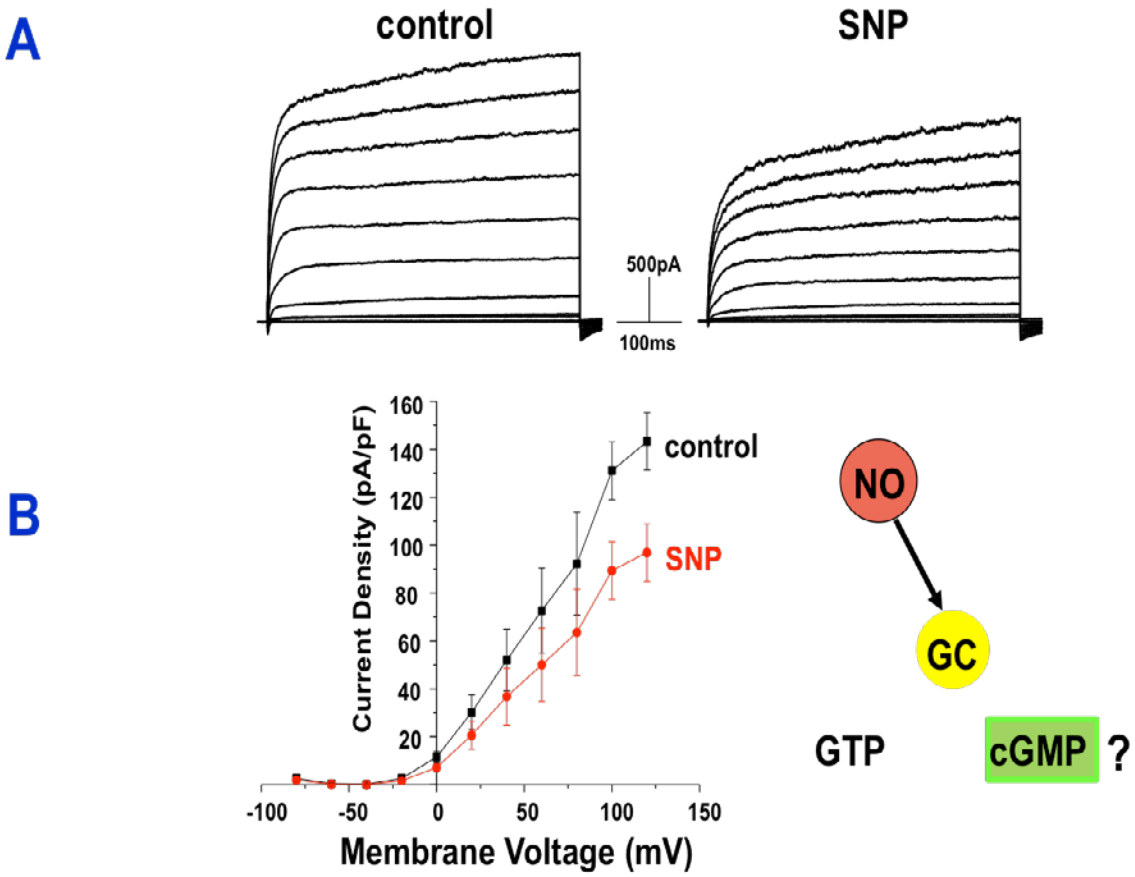

Figure 5-2

Figure 5-3: Sodium nitroprusside (SNP; NO donor) reduces K_{DR} in astrocytes. **(A)** Membrane Currents (-80 to +120 mV; Vhold = -50 mV) recorded from the same astrocyte before (*control*) and 15 min after exposure to 10 μM SNP. **(B)** Complete average current-voltage relationship for steady-state outward currents as in A. Each point represents the mean of 3 cells ± S.E. On average, SNP decreased K_{DR} current density by 33.3 ± 6% (+80 mV). Proposed signaling pathway for SNP / NO is illustrated to the right.

III. cGMP decreases K_{DR} Currents In Astrocytes

As illustrated in Figure 5-4, increasing cGMP levels in astrocytes depressed currents carried by K_{DR} channels. Tracings provided in Figure 5-4A are recorded from the same astrocyte (V_{hold} -50mV) before and after exposing the cell to 500 μM 8Br-cGMP (20 min). Increasing intracellular cGMP levels decreased steady-state outward currents at every test potential. On average, 8Br-cGMP decreased steady-state K_{DR} currents 15 $\pm$ 3% (+80mV; n=3). A summary normalized current – voltage relationship of experiments from 3 astrocytes, before and after exposure to 8Br-cGMP is illustrated in Figure 5B.

Elevation of cellular cGMP levels is known to stimulate at least 3 prominent effector molecules in a variety of cell types: cGMP-gated ion channels, phosphodiesterases, and the cGMP-dependent protein kinase (PKG). Previous studies from our laboratory had measured powerful effects of PKG on outward potassium currents in vascular smooth muscle cells (White et al., 1995; White et al., 2000). Because we had observed depression of potassium currents by treating astrocytes with either NO or cGMP, we next tested the possibility that cGMP was decreasing K_{DR} currents in astrocytes via cGMP-dependent phosphorylation mediated by PKG.

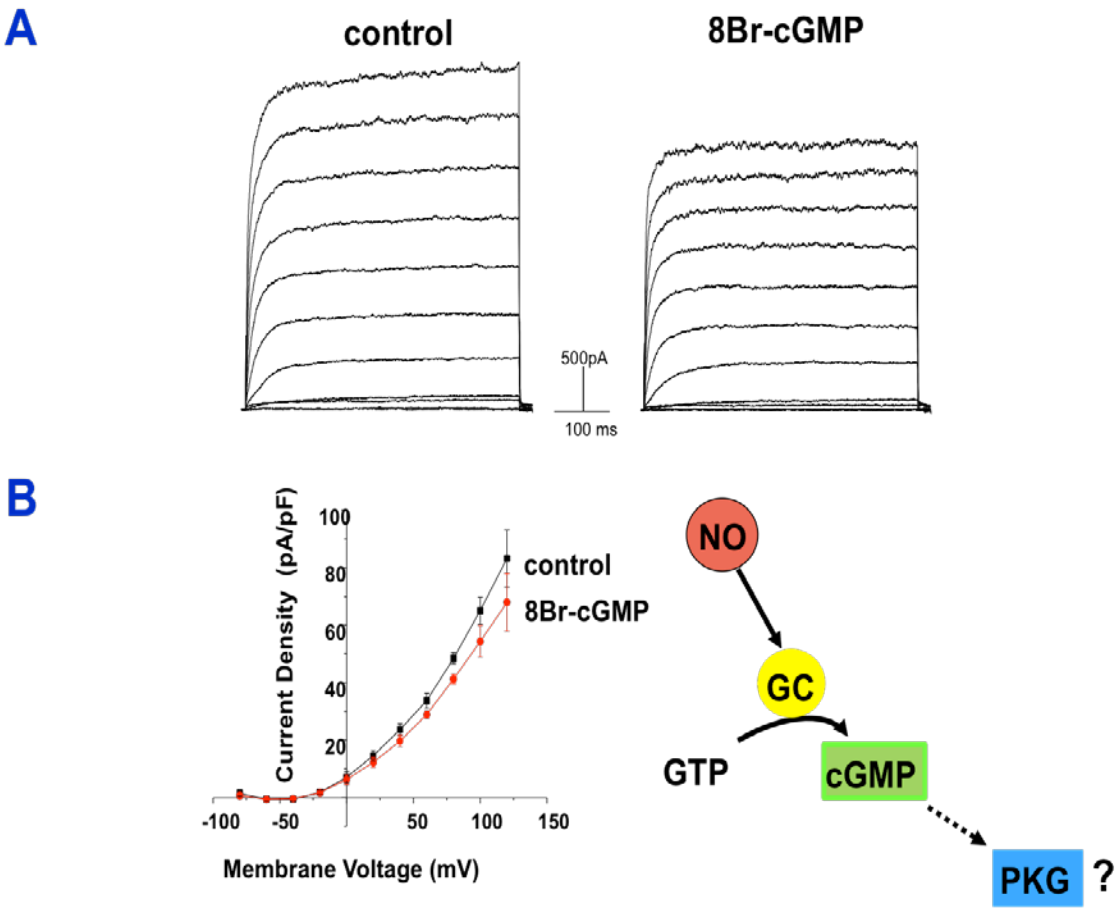

Figure 5-3

Figure 5-4. Elevating intracellular cGMP decreases K_{DR} currents in astrocytes. **(A)** Membrane currents (-80 to +120 mV; Vhold = -50 mV) recorded from the same astrocyte before (control) and 20 min after exposure to 500 μM 8Br-cGMP, a membrane-permeable cGMP derivative. **(B)** Complete average current density - voltage relationship for steady-state outward currents as in A. Each point represents the mean of 3 cells ± S.E. On average, 8Br-cGMP decreased K_{DR} current density by 15 ± 3% (+80mV). Proposed signaling pathway for cGMP is illustrated to the right.

IV. Inhibition of PKG attenuates cGMP-induced inhibition of KDR currents

As expected, treating astrocytes with 500 μM 8Br-cGMP depressed steady-state K_{DR} currents in astrocytes (Figure 5-5). However, subsequent cumulative addition of 300 nM KT5823, a selective inhibitor of PKG activity, completely reversed the inhibitory effect of cGMP on outward currents. Moreover, inhibition of PKG activity increased the amplitude of K_{DR} currents well above control levels. In fact, inhibition of PKG activity with KT5823 more than doubled K_{DR} currents at all test voltages. These findings not only identified PKG as the primary downstream mediator of cGMP action on membrane currents in astrocytes, but also strongly suggested that PKG exerts a tonic suppression of K_{DR} current under basal (non-stimulated) conditions – and thus help maintain astrocyte excitability.

Experiments in other cell types had established that PKG, although activated by cGMP preferentially, is not specific for cGMP alone. In fact, previous findings from our laboratory had demonstrated that cAMP can cross-activate PKG and thereby modulate cellular potassium currents (White et al., 2000; Zhu et al., 2002). Thus, it was possible that PKG activity in astrocytes might also be stimulated by cAMP, another powerful and ubiquitous cellular second messenger.

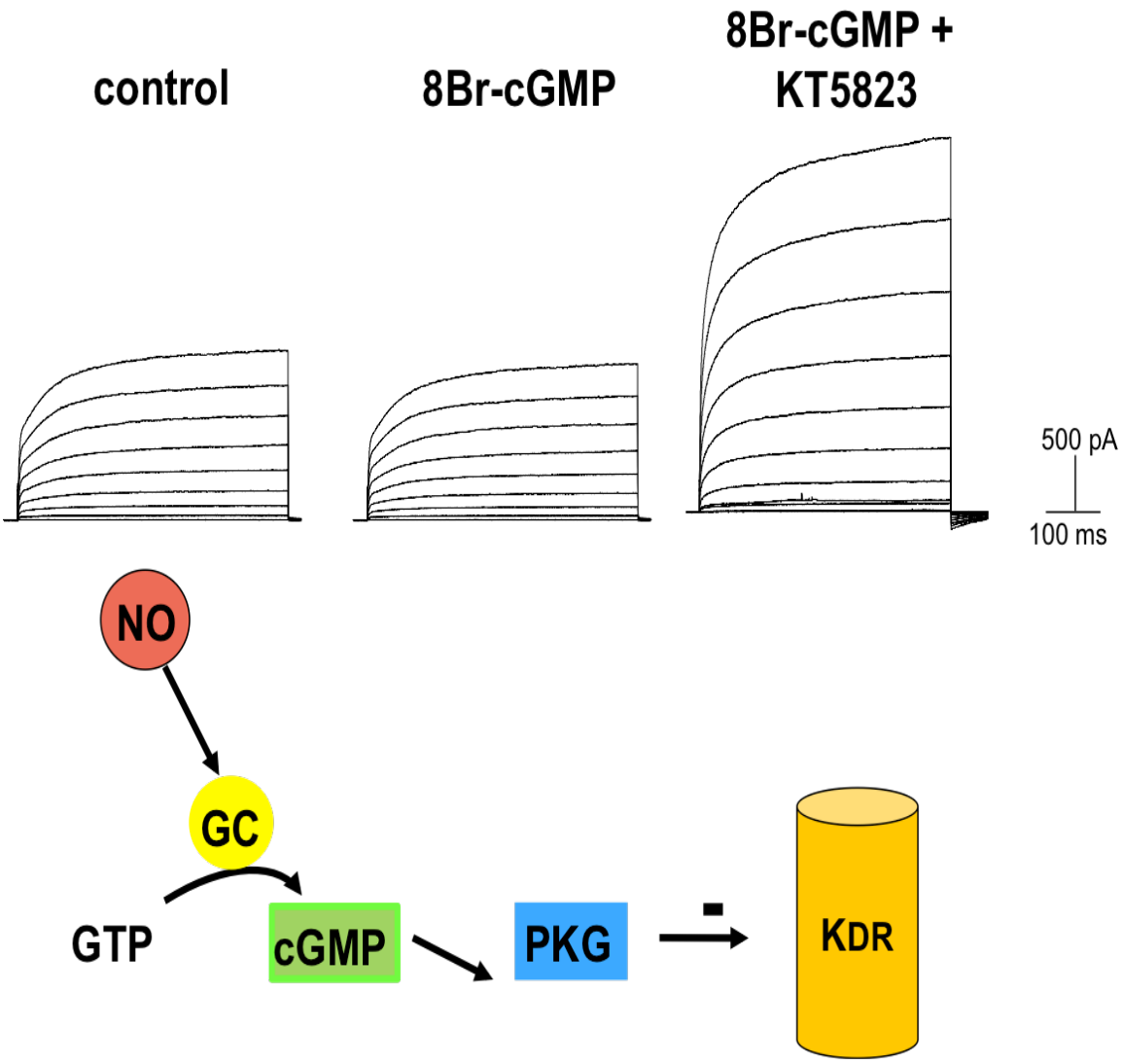

Figure 5-4

Figure 5-5: Inhibiting PKG activity reverses the stimulatory effect of cGMP on K_{DR} currents in astrocytes. Top panel illustrates outward currents recorded in the same astrocyte (V_{hold} -50mV) before (control), 20 min after 500 μM 8Br-cGMP, and then 30 min after further addition of 300nM KT5823 (a selective PKG inhibitor). Not only did inhibition of PKG reverse the inhibitory effect of cGMP, but KT5823 more than doubled outward currents in this cell.

IV. Cyclic AMP Decreases K_{DR} Currents In Astrocytes

To test the possibility that cAMP might also suppress K_{DR} currents in astrocytes, we performed another series of experiments in which we generated cAMP in these cells and measured outward currents. As illustrated in Figure 5-6A, addition of 10 μM forskolin (20 min), a stimulator of adenylyl cyclase activity, decreased outward currents in astrocytes at all test voltages. This inhibitory effects was further illustrated by the summary current density – voltage relationship for steady-state outward (K_{DR}) currents in astrocytes. On average, forskolin decreased K_{DR} current density by 29.8 $\pm$ 6% (+80mV; n=3 cells). These findings strongly suggest that K_{DR} currents in astrocytes are regulated by both cGMP and cAMP; however, the signaling pathways downstream from these nucleotides may then converge on a common effector molecule, PKG, which mediates suppression of K_{DR} currents most likely by phosphorylation of K_{DR} channels or a closely-associated regulatory protein.

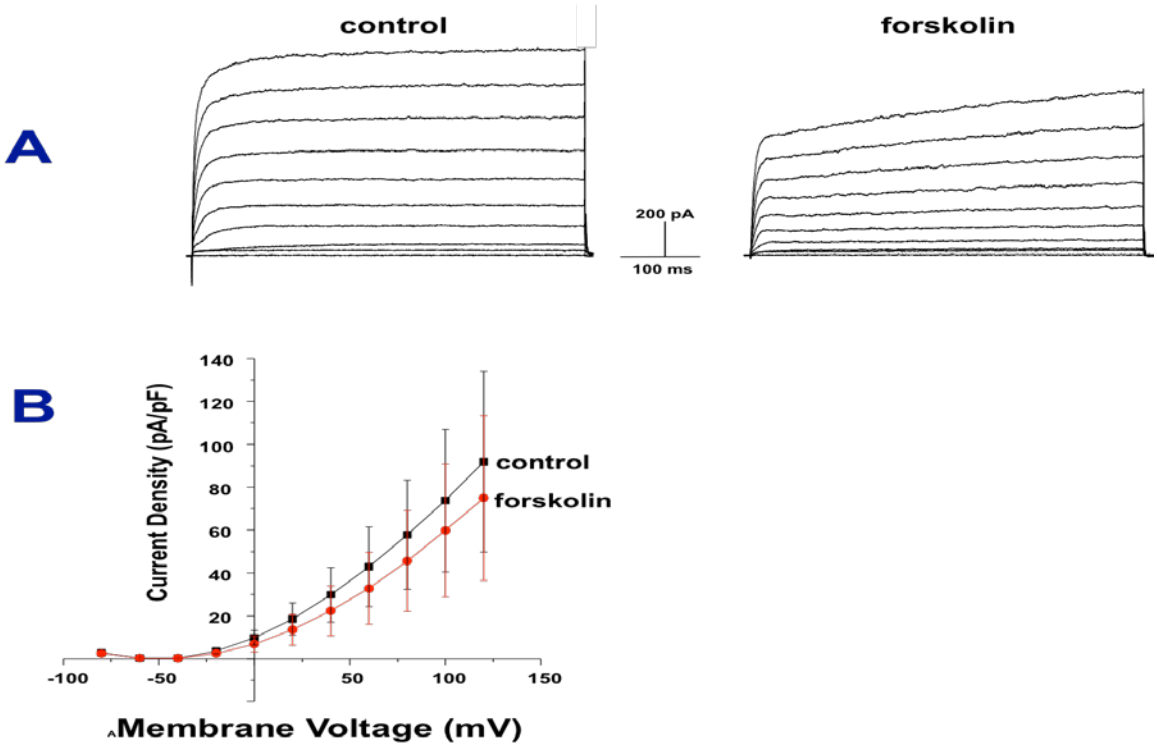

Figure 5-5

Figure 5-6. Elevation of cAMP depresses K_{DR} currents in astrocytes.

(A) Membrane currents (-80 to +100 mV; Vhold = -50 mV) from the same astrocyte before (control) and 20 min after exposure to 10 μM forskolin, a cAMP agonist. **(B)** Complete summary current density-voltage relationship for steady-state outward currents as in A. Each point represents the mean of 3 cells ± S.E. On average, forskolin decreased K_{DR} current density by 29.8 ± 6% (+80 mV).

6. Discussion

The incidence of neurological disease is increasing, and yet there is no cure for most diseases with debilitating loss of memory and brain function. Astrocytes protect neuronal function, and are a possible therapeutic target for helping to slow the progression of neurological diseases. However, we know relatively little about the molecular basis of astrocyte protection in most neurological diseases. Over the past decade there has been increasing evidence that cGMP is an important secondary messenger in the brain and is modified by different physiological and pathological conditions in certain areas of the brain. Recent clinical trials indicate that phosphodiesterase inhibitors (e.g., sildenafil) may improve learning and memory by increasing the levels of cGMP in the brain, as does increasing levels of nitric oxide. But at the present time, the mechanisms whereby the NO/cGMP/PKG signaling pathway can help alleviate memory loss and cognitive function are unknown. Our findings suggest that increases in cGMP via nitric oxide signaling depress steady-state outward potassium currents in astrocytes via cGMP-dependent phosphorylation mediated by PKG. We believe this mechanism helps to improve normal synaptic neurotransmission. Through electrophysiology, using the perforated whole-cell patch clamp technique, we have identified a potential novel target of cGMP action in astrocytes: the delayed-rectifier potassium (K_{DR}) channel.

Astrocytes exhibit inward currents via sodium channels and both inward and outward currents via potassium channels; however, these cells do not

propagate action potentials (Sofroniew et al., 2010*).* Nonetheless, astrocytes are excitable via regulated increases in intracellular calcium concentrations. Evidence shows that increases in intracellular calcium are of functional significance in astrocyte-astrocyte and astrocyte-neuron intercellular communication (Sofroniew et al., 2010). Increases in cytosolic calcium concentrations can result from the release of intracellular calcium stores, neurotransmitters released during neuronal activity, or by propagation of signals from neighboring astrocytes (Sofroniew et al., 2010). Astrocytes envelop all synapses, provide essential functions that maintain the homeostasis of synaptic transmission, and play direct roles in synaptic transmission through the release of gliotransmitters in response to neuronal synaptic activity. (Sonfroniew et al., 2010). Astrocytic mechanisms exert powerful influences on synaptic remodeling in the CNS or in response to CNS disorders (Sofroniew et al., 2010).

Gliotransmitters are released in response to astrocytic excitability via increases in intracellular calcium concentrations. Astrocytes express L-type calcium channels, which exhibit an activation threshold of approximately -35mV (Bijlani et al., 2010). Thus, relatively small depolarizing stimuli can enhance calcium channel opening and stimulate astrocyte excitation and release of gliotransmitters. In addition to calcium channels, potassium channels exert a powerful influence upon astrocyte membrane potential, and as such are a major regulatory influence upon calcium channel gating. Astrocytes express several species of voltage-dependent potassium channels, with K_{DR} being a prominent

example. Depolarization increases the activity of K_{DR} channels, and their repolarizing influence serves as a "brake" on cellular activity by closing voltage-dependent calcium channels and thereby depressing gliotransmission. Conversely, any agent that decreases currents through K_{DR} channels (e.g., neurotransmitters, second messengers, drugs) would tend to enhance cellular depolarization, and thereby stimulate gliotransmission.

NO is a liposoluble gaseous molecule which readily passes through the cellular membrane. NOS are the enzymes responsible for the formation of NO, and exist in three isoforms: iNOS, eNOS, nNOS. NO is involved in almost all processes of neuronal transmission due to the ubiquitous distribution of NOS throughout the brain, where its primary function is to activate sGC. sGC when activated by NO catalyzes the conversion of GMP to cGMP, which in turn activates PKG. It is PKG that functions as a primary molecular effector by phosphorylating a variety of signaling substrate molecules in the brain, and thereby modifying their function. PKG is known to be a critical factor during synaptic plasticity. Therefore, increases of NO via NOS lead to increases of intracellular cGMP and PKG activity, and thereby likely modulate synaptic plasticity.

Much research has been focused on cGMP due to its ability to improve memory and cognitive function. For example, a recent and promising therapeutic development is the use of PDE inhibitors to enhance memory by increasing cGMP levels in the brain. The NO/cGMP pathway is the one of the physiological mediums for producing cGMP in the cell, yet our understanding of

how NO/cGMP modulates excitability of neurons and astrocytes is minimal, at best. To help address this gap in our knowledge, we measured the effects of NO/cGMP on cellular currents in astrocytes directly. We initially elevated NO levels in isolated astrocytes by administering sodium nitroprusside, which releases NO as it is metabolized by cells. Our findings demonstrated that NO depressed outward potassium currents carried predominately via the K_{DR} channel. This depression of K_{DR} current would suppress efflux of potassium ions, thereby exerting a depolarizing (excitatory) influence on astrocyte activity. Thus, NO would promote depolarization-stimulated increased calcium accumulation and enhanced excitability. Since cGMP production is a downstream effector of NO, our next question was: Would adding cGMP directly mimic the effect of NO on K_{DR}?

cGMP plays a role in the biochemical cascade that regulates cognitive functions, and is assumed to mediate the positive therapeutic effect of PDE inhibitors. PDEs regulate cyclic nucleotide (cGMP and/or cAMP) signaling by metabolizing and inactivating these powerful intracellular second messengers (Zhihui et al., 2013). Thus, PDE inhibitors increase cellular cGMP concentrations, but do so independent of NO generation (Zhihui et al., 2013). PDE inhibitors increase cGMP concentrations, which may trigger downstream phosphorylation via PKG.

Studies have shown that aging increases PDE expression and decreases cGMP concentrations in the brain (Zhihui et al., 2013). There is also substantial evidence that cGMP plays a part in signaling cascades that are important for

learning and memory. Recent studies imply that long-term memory may be enhanced significantly in patients given cGMP-targeting PDE inhibitors (Bollen et al., 2014). cGMP-specific PDE inhibitors serve as a modulatory function for memory and synaptic plasticity. Development of specific PDE inhibitors has been suggested as novel therapeutic targets for certain neurological diseases that cause loss of memory and cognitive function. However, the mechanistic action for how this is accomplished is not understood. Our findings now provide evidence for a novel cellular mechanism that could potentially account for the therapeutic effect of PDE inhibitors on memory: depression of K_{DR} currents leading to enhanced excitation of astrocytes (and possibly neurons as well).

It is of importance to determine the effects of increased intracellular cGMP concentrations on K_{DR} to further our understanding of the mechanism of how NO/cGMP/PKG signaling enhances learning, memory, and cognitive function. 4 did in fact depress K_{DR} currents. These findings remain consistent with our hypothesis, and were confirmed by our findings that increasing NO depressed K_{DR} in astrocytes. Although the direct effect of cGMP (i.e., 8Br-cGMP) on K_{DR} currents was not as robust as the effect of sodium nitroprusside (Figure 6), this difference is likely related to the difficulty of cGMP to permeate the cell membrane – unlike NO, which generates cGMP within astrocytes more directly via activation of sGC. Nonetheless, the depression of K_{DR} was statistically significant and important to the overall findings. The data clearly demonstrate

that raising intracellular concentrations of cGMP either directly with 8Br-cGMP or indirectly with NO decreases K_{DR} in embryonic astrocytes.

Most researchers believe that object memory and cognitive function are dependent on cGMP-dependent phosphorylation (i.e., PKG activity) in the hippocampus. Indeed, studies suggest that cGMP and PKG may facilitate memory formation and synaptic plasticity (Bollen et al., 2014). Interestingly, cGMP-PKG signaling has shown to act as a modulatory system that increases Ca^{2+} in neurons. We believe that this same signaling system may intrinsically modulate Ca^{2+} influx in astrocytes, and propose that depression of K_{DR} currents may be an important mechanism that produces this response.

Previous studies suggest that elevated levels of cGMP and PKG can lower stimulation threshold for Ca^{2+} signals (Bollen et al., 2014). However, the mechanism for how this is done is not understood. To test our hypothesis that PKG mediated the inhibitory effect of NO/cGMP on K_{DR} currents, we employed an antagonist (KT5823) that exhibits high selectivity for PKG. Our findings clearly demonstrated that inhibition of PKG activity attenuated the inhibitory effect of cGMP on K_{DR}, thus indicating that PKG-mediated phosphorylation depresses activity of K_{DR} channels in embryonic astrocytes. Somewhat surprisingly, KT5823 increased the amplitude of outward far above basal levels, suggesting that PKG likely exerts a tonic inhibitory influence on K_{DR} currents even under non-stimulated conditions. As discussed above, this inhibitory influence of PKG on potassium efflux – during stimulated or non-stimulated conditions -- would help maintain astrocyte depolarization, prolong calcium

influx, and thereby stimulate gliotransmission – leading to enhanced synaptic plasticity. In sum, we propose that cGMP-dependent phosphorylation is an important intrinsic signaling mechanism that enhances synaptic plasticity, memory and cognitive function via inhibition of the K_{DR} currents. Further, these findings suggest that K_{DR} channels and cGMP signaling molecules are likely to be powerful therapeutic targets for helping to treat Alzheimer's and possibly other debilitating neurological diseases.

To our knowledge, these findings are the first to demonstrate an inhibitory effect of PKG on K_{DR} currents in astrocytes, and are supported by previous studies indicating that PKG-mediated phosphorylation modulates K_{DR} currents in other cell types. For example, Shimuzi et al. (2002) suggested that K_{DR} is decreased by PKG in guinea-pig sinoatrial (SA) node cells. In addition, Xu et al. (2008) found that 8-Br-cGMP inhibited K_{DR} in both hypoxic and normal rat pulmonary artery smooth muscle cells. As we found in astrocytes, inhibition of PKG with KT5823 completely blocked the inhibition of K_{DR} via 8-Br-cGMP in these muscle cells. Thus, it was suggested that the effect of cGMP on K_{DR} is mediated by PKG-dependent phosphorylation of the channel. At present it is unknown whether the K_{DR} channel protein is the actual substrate for PKG-dependent phosphorylation, or whether PKG might instead phosphorylate a closely-associated regulatory protein. However, it is speculated that phosphorylation of K_{DR} channel proteins by PKG might alter the voltage-sensitivity of the channel (Shimuzi et al. 2002). K_{DR} possesses four phosphorylation-sites for the cAMP-dependent kinase (PKA), and exhibits cyclic

nucleotide binding domains in the C-terminal that can be modulated via the cAMP-PKA phosphorylation pathway in guinea pig ventricular myocytes (Xu et al., 2008). Because PKG, like PKA, is a serine-threonine kinase, it is possible that similar consensus phosphorylation sites for PKG exist on the K_{DR} channel protein; however, future studies are needed to identify these putative PKG phosphorylation sites on K_{DR}. Of note, we also found that increasing cAMP levels in astrocytes (via forskolin) decreased K_{DR} currents, quite possibly via cross-activation of PKG. Although a potential role for PKA in astrocyte excitability has not yet been established, elevating cAMP by treatment with the PDE inhibitor rolipram enhanced synaptic and cognitive function in a mouse model of AD (Gong et al., 2004.). Thus, it is apparent that elevation of cyclic nucleotide levels (either cGMP or cAMP) is a promising treatment for AD and possibly other disorders of memory and cognition. Our findings are the first to propose a single unifying signaling mechanism where by both cyclic nucleotides may act to enhance brain function: phosphorylation of K_{DR} channels in astrocytes via activity of the cGMP-dependent protein kinase.

6. Conclusion and Future Studies

Our research demonstrates that currents through K_{DR} channels dominate membrane electrical activity in embryonic astrocytes. We found that NO/cGMP signaling depresses these K_{DR} currents, and that the effects of NO/cGMP are likely mediated by cGMP-dependent phosphorylation of the K_{DR} channel (or a closely-associated regulatory protein). This depolarizing effect would prolong opening of voltage-dependent, L-type calcium channels to elevate astrocyte calcium levels and enhance gliotransmission. Increased gliotransmission would be likely to enhance synaptic plasticity, which may lead to improved memory and cognitive function. These findings thus provide the first evidence for a potential molecular mechanism that could now explain the beneficial effect of PDE inhibitors and other cGMP–enhancing agents on learning and memory.

Though important, a limitation of these studies is that they consist of electrophysiological studies of embryonic astrocytes. Future studies are needed to examine the effects of enhanced cGMP-dependent phosphorylation of the K_{DR} via PKG in adult astrocytes. Future electrophysiologic studies will also be needed to explore potential effects of increased intracellular concentrations of cGMP on L-type calcium channels in adult astrocytes to ensure that the phosphorylation of K_{DR} channels is indeed causing calcium channels to open and remain open to cause gliotransmission via astrocytic excitability.

7. References

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